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NOTE

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Extractives of *Juniperus chinensis* L. I: Isolation of podophyllotoxin and yatein from the leaves of *J. chinensis*

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Abstract Two compounds, yatein and podophyllotoxin, were isolated from the chloroform-soluble fraction in the methanolic extractives of byakushin (*Juniperus chinensis* L.) leaves for the first time.

Key words *Juniperus chinensis* · Extractives · Lignans · Podophyllotoxin · Yatein

Introduction

This study is a continuation of our work on the production of biologically active substances by tissue cultures of trees[1,2] and the microbial and enzymatic conversion of extractives from trees.[3–7] We investigated the extractives of byakushin (*Juniperus chinensis* L.) in search of potential compounds for biotechnological production of biologically active substances.

Juniperus chinensis is an evergreen tree with scaly, partly needle, year-round foliage. It is grown in Japan, Korea, and China.[8] To date, only two studies have examined extractives from the leaves of this plant. One study, conducted by Sawada,[9] isolated two bisflavonoids (hinokiflavone and kayaflavone). The other study, by Fang et al.,[10] isolated a counmarin (umbelliferone), 13 lignans, and 2-arylpropane-1,3-diol. Fang et al. did not isolate podophyllotoxin or yatein from the leaves of kaizukaibuki (*J. chinensis var.* Kaizuka Hort.), a variety of byakushin.

Podophyllotoxin, a lignan with strong antileukemic and tumor-inhibiting effects, has been isolated from the leaves of *Juniperus sabina*, along with several podophyllotoxin-related compounds.[11] It is believed that podophyllotoxin and related compounds are contained in *J. chinensis* leaves. Podophyllotoxin is a pharmaceutically valuable compound that has become commercially important as raw material for the semisynthesis of the antitumor agents etoposide and teniposide. Natural sources of podophyllotoxin are mainly species of *Podophyllum*, but a search for a new source of podophyllotoxin other than species of *Podophyllum* is required because large amounts of podophyllotoxin are needed as raw material for production of the antitumor agents. Isolation of podophyllotoxin and related compounds from the leaves of *J. chinensis* has not yet been reported. We describe herein the isolation of podophyllotoxin and yatein, a compound related to podophyllotoxin, from the leaves of *J. chinensis*.

Results and discussion

In the present study of *J. chinensis* leaves, two compounds were isolated: yatein (compound 1) and podophyllotoxin (compound 2). This is the first time that compounds (1) and (2) have been found in these sources. Their chemical structures are shown in Fig. 1. The structures were determined by ultraviolet (UV), proton nuclear magnetic resonance (^{1}H-NMR), and carbon-13 nuclear magnetic resonance (^{13}C-NMR) spectroscopy and mass spectrometry (MS).

Compound (1) was isolated as a colorless oil, $C_{22}H_{24}O_7$, $[\alpha]^{25}_D$-25.0° ($CHCl_3$), whose molecular ion peak (M^+) was observed at m/z 400 in the mass spectrum. The UV spectrum of compound (1) had bands at $\lambda^{CHCl_3}_{max}$: 287 and 242 nm and showed a characteristic UV absorption spectrum of dibenzylbutyrolactone lignans.[12] The mass spectrum of the compound agreed well with the proposed structure.[13] No acetate was formed on acetylation of compound (1) with acetic anhydride and pyridine, which showed that compound (1) had no free phenolic or primary and secondary alcoholic hydroxyl groups. In the ^{1}H-NMR spectrum of the compound, signals of a 1,3,4-tri-substituted aromatic ring at 6.46, 6.47, and 6.69 ppm, a 1,3,4,5-tetra-substituted aromatic ring at 6.36 ppm, and two protons of a methylenedioxy at

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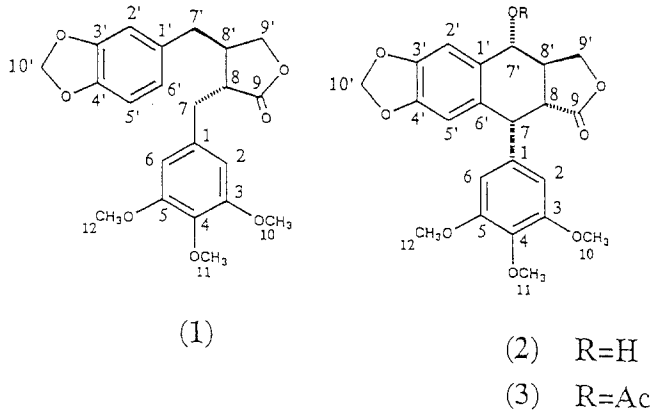

Fig. 1. Yatein (1) and podophyllotoxin (2) isolated from the leaves of *Juniperus chinensis* L.

5.93 and 5.94 ppm were observed. Furthermore, signals of four benzyl protons attached at the C-7′ and C-7 positions at 2.48–2.63 ppm and 2.89–2.92 ppm, respectively, two protons of a methylene attached to the lactone oxygen at 4.18 and 3.88 ppm, two *trans*-protons attached at the C-8 and C-8′ positions at 2.48–2.63 ppm, and three methoxyl groups at 3.82 ppm were also observed in the spectrum. MS and ^{1}H-NMR spectra of compound (1) were in good agreement with those of authentic samples of yatein isolated from *Podophyllum hexandrum*.[14] The ^{13}C-NMR spectrum well explained the structure of yatein.[15] The ^{13}C-NMR assignments are shown in Table 1. Furthermore, the specific rotation ($[\alpha]_D$) of compound (1) agreed with that of yatein isolated from *Libocedrus yateensis*.[16] Therefore, compound (1) was identified as yatein and was obtained in a yield of 0.0008% of the fresh leaves.

Compound (2) was isolated as white crystals, $C_{22}H_{22}O_8$, melting point (mp) 181°–183°C, $[\alpha]^{25}_D$-121.3° ($CHCl_3$), whose M^+ was observed at m/z 414 in the mass spectrum. The UV spectrum of compound (2) had bands at λ^{EtOH}_{max}: 291 and 219 nm. The mass spectrum of compound (2) agreed well with the proposed structure.[17] In the ^{1}H-NMR spectrum of the compound, signals of a 1,2,4,5-tetra-substituted aromatic ring at 6.51 and 7.11 ppm, a 1,3,4,5-tetra-substituted aromatic ring at 6.37 ppm, two protons of a methylenedioxy at 5.97 and 5.99 ppm, two methoxyl groups at 3.76 ppm, and a methoxyl group at 3.81 ppm attributed to the substituents at the C-3 and C-5 positions and the C-4 position, respectively, were observed. Furthermore, signals of two benzyl protons attached at the C-7 and C-7′ positions at 4.62 and 4.77 ppm, two protons of a methylene attached to the lactone oxygen at 4.08 and 4.59 ppm, two *trans*-protons attached at the C-8 and C-8′ positions at 2.7–2.9 ppm, and a proton of a hydroxyl group attached at the C-7′ position at 2.17 ppm were also observed in the spectrum. MS and ^{1}H-NMR spectra of compound (2) were in good agreement with those of authentic samples of podophyllotoxin purchased from Sigma Chemical Company.[17] The ^{13}C-NMR spectrum well explained the structure of the compound.[15] The ^{13}C-NMR assignments for compound (2) are shown in Table 1. The specific rotation ($[\alpha]_D$) of compound (2) agreed with that of podophyllotoxin,[18] which was isolated from the podophyllin N.F. prepared from *Podophyllum peltatum*.[19]

Table 1. ^{13}C-NMR assignments for yatein and podophyllotoxin (100 MHz, $CDCl_3$, TMS as internal standard)[a]

Carbon[b]	Yatein	Podophyllotoxin
C-1	133.3	135.4
C-2	106.3	108.5
C-3	153.2	152.6
C-4	136.8	137.3
C-5	153.2	152.6
C-6	106.2	108.5
C-7	38.3	44.1
C-8	46.4	45.3
C-9	178.5	174.4
C-10	56.1	56.3
C-11	60.8	60.8
C-12	56.1	56.3
C-1′	131.5	133.1
C-2′	108.7	106.3
C-3′	147.9	147.7
C-4′	146.4	147.8
C-5′	108.3	109.8
C-6′	121.5	131.2
C-7′	35.2	72.8
C-8′	41.0	40.8
C-9′	72.0	71.3
C-10′	101.1	101.5

[a] ^{13}C chemical shifts are ppm from tetramethylsilane (TMS).
[b] For numbering of carbons in yatein and podophyllotoxin, refer to Fig. 1

On acetylation of compound (2) with acetic anhydride and pyridine, crystalline monoacetate (3), mp 207°–209°C, was obtained. The ^{1}H-NMR and MS spectra of compound (3) were in good agreement with those of authentic samples prepared from podophyllotoxin. The ^{13}C-NMR spectrum of compound (3) coincided with that of authentic podophyllotoxin monoacetate.[15] The mixed-melting-point test of compound (3) with authentic podophyllotoxin monoacetate proved the compound (3) to be identical with podophyllotoxin monoacetate. Therefore, compound (2) was identified as podophyllotoxin and was obtained in a yield of 0.00009% of the fresh leaves.

Podophyllotoxin and its related compound yatein were isolated for the first time from the leaves of *J. chinensis*. Podophyllotoxin has strong antileukemic and tumor-inhibiting activities.[10] Several studies have indicated that podophyllotoxin is contained in the leaves of *Juniperus sabina* (genus *Juniperus*, Cupressaceae)[11] and in the rhizomes of *Podophyllum* species.[17,21] The amounts of podophyllotoxin in *J. sabina* and *J. chinensis* are small, but species of *Podophyllum* produce large amounts of this lignan in their roots. Podophyllotoxin content is considered to be increased by tissue cultures of *J. chinensis*. We found that the content of podophyllotoxin could be increased by about 15 times that of the intact plant by callus cultures of *J. chinensis*.[22] Production of podophyllotoxin by callus cultures and cell suspension cultures of *J. chinensis* are now being conducted.

Experimental

Plant material

Fresh leaves (mixture of scaly and partly needle foliage) of *J. chinensis* were collected in May 1996 in Uwajima City, Ehime Prefecture.

Extraction from *J. chinensis* leaves

Extraction from fresh leaves of *J. chinensis* (17 kg) was carried out twice for 4 days with methanol at room temperature. The methanolic extractives (1.09 kg) were suspended with water and successively extracted with *n*-hexane, chloroform, ethyl acetate, and *n*-butanol. The chloroform-soluble fraction gave a positive color reaction of *Podophyllum* lignans[20] with acetic acid and concentrated nitric acid (10:3 v/v) on a thin-layer chromatography (TLC) plate.

Isolation of compounds (1) and (2) from chloroform-soluble fraction

The chloroform-soluble fraction (64.8 g) was separated into two fractions (fractions 1 and 2) by silica gel column chromatography with a chloroform-methanol stepwise elution. Fractions 1 and 2 were eluted out, in order. Fraction 1 was chromatographed on a silica gel column using a benzene-ethyl acetate stepwise elution and yielded fraction 1-1, containing a lignan (291 mg). Fraction 1-1 was rechromatographed on a preparative thin-layer chromatography (PTLC) with chloroform-acetone (65:35 v/v) and yielded compound (1) (133 mg).

Fraction 2 was chromatographed on a silica gel column using chloroform-ethyl acetate stepwise elution followed by benzene-ethyl acetate stepwise elution to afford fraction 2-1, containing a lignan (192 mg). Fraction 2-1 was rechromatographed on PTLC with chloroform-acetone (65:35 v/v) to give crude compound (2) (56.6 mg).

Yatein

Compound (1) (yatein) was obtained as a colorless oil. *Analysis.* Calculated: $C_{22}H_{24}O_7$: C, 65.97; H, 6.04. Found: C, 65.82; H, 6.14. $[\alpha]^{20}_D = -25.0°$ ($c = 0.02$ in $CHCl_3$). {lit $[\alpha]^{20}_D = -28.4°$ ($c = 0.32$ in $CHCl_3$)}.[16] UV $\lambda^{CHCl_3}_{max}$ nm ($\log\varepsilon$): 242 (3.75), 287 (3.54). MS m/z (mass/charge ratio peaks): 400 (M^+), 265, 264, 251, 238, 219, 182, 181 (100%), 167, 135, 131, 121, 77. ^{1}H-NMR (400 MHz, $CDCl_3$) δ (chemical shift): 2.48–2.63 [(4H (protons), *m* (multiplet), 2 × H7′, H8′, and H8)], 2.89–2.92 (2H, *m*, 2 × H7), 3.82 [(9H, *s* (singlet), 3 × OMe)], 3.88 {[1H, *dd* (double doublet), *J* (coupling constant) = 9.3, 7.8 Hz, H9′]}, 4.18 (1H, *dd*, *J* = 9.3, 7.3 Hz, H9′), 5.93 [(1H, *d* (doublet), *J* = 1.5 Hz, H10′)], 5.94 (1H, *d*, *J* = 1.5 Hz, H10′), 6.36 (2H, *s*, H2 and H6), 6.46 (1H, *d*, *J* = 1.7 Hz, H2′), 6.47 (1H, *dd*, *J* = 6.9, 1.7 Hz, H6′), 6.69 (1H, *d*, *J* = 8.3 Hz, H5′). The ^{13}C-NMR data are shown in Table 1. ^{1}H-NMR and ^{13}C-NMR data of compound (1) were identical with those of authentic yatein.[14,15]

Podophyllotoxin

The compound (2) (podophyllotoxin) obtained from fraction 2-1 was recrystallized from chloroform and ethanol to afford white crystals (2) (14.7 mg), mp 181°–183°C (lit mp 180.8°–181.8°C).[21] Compound (2) was obtained in a yield of 0.00009% of the fresh leaves. *Analysis.* Calculated: $C_{22}H_{22}O_8$; C, 63.75; H, 5.35. Found: C, 63.89; H, 5.36. $[\alpha]^{25}_D = -121.3°$ ($c = 0.03$ in $CHCl_3$) {lit $[\alpha]^{20}_D = -132°$ ($c = 1.0$ in $CHCl_3$)}.[18] UV λ^{EtOH}_{max} nm ($\log\varepsilon$): 219 (4.51), 291 (3.89). MS m/z: 414 (M^+) (100%), 399, 396, 189, 181, 168, 153. ^{1}H-NMR (400 MHz, $CDCl_3$) δ: 2.17 (1H, *d*, *J* = 8.3 Hz, OH), ca 2.7–2.9 (2H, *m*, H8 and H8′), 3.76 (6H, *s*, 2 × OMe), 3.81 (3H, *s*, OMe), 4.08 (1H, *t* (triplet), *J* = 9.5 Hz, H9′), 4.59 (1H, *m*, H9′), 4.62 (1H, *d*, *J* = 8.8 Hz, H7), 4.77 (1H, *t*, *J* = 8.8 Hz, H7′), 5.97 (1H, *d*, *J* = 1.5 Hz, H10′), 5.99 (1H, *d*, *J* = 1.5 Hz, H10′), 6.37 (2H, *s*, H2 and H6), 6.51 (1H, *s*, H5′), 7.11 (1H, *s*, H2′). The ^{13}C-NMR data are shown in Table 1. The ^{13}C-NMR spectra of compound (2) were identical with those of authentic podophyllotoxin.[15]

Podophyllotoxin monoacetate

Crude compound (2) (20 mg) was acetylated with acetic anhydride (1 ml) in pyridine (1 ml), and the workup was conducted in the usual manner to afford monoacetate (3) (20.1 mg) after recrystallization with ethanol, mp 207°–209°C (lit mp 206°–207°C).[23] *Analysis.* Calculated: $C_{24}H_{24}O_9$: C, 63.14; H, 5.30. Found: C, 63.04; H, 5.39. UV λ^{MeOH}_{max} nm ($\log\varepsilon$): 209 (4.87), 278 (4.29). MS m/z: 456 (M^+) (100%), 397, 351, 282, 229, 185, 168, 149. ^{1}H-NMR (400 MHz, $CDCl_3$) δ: 2.19 (3H, *s*, OAc), ca. 2.8–2.9 (2H, *m*, H8 and H8′), 3.76 (6H, *s*, 2 × OMe), 3.81 (3H, *s*, OMe), 4.20 (1H, *t*, *J* = 9.7 Hz, H9′), 4.39 (1H, *dd*, *J* = 9.3, 6.8 Hz, H9′), 4.61 (1H, *d*, *J* = 4.4 Hz, H7), 5.88 (1H, *d*, *J* = 8.8 Hz, H7′), 5.98 (1H, *d*, *J* = 1.5 Hz, H10′), 6.0 (1H, *d*, *J* = 1.5 Hz, H10′), 6.39 (2H, *s*, H2 and H6), 6.54 (1H, *s*, H5′), 6.77 (1H, *s*, H2′). ^{13}C-NMR (100 MHz, $CDCl_3$) δ: 21.1 (—O—CO—*CH*$_3$), 38.7 (C8′), 43.7 (C7), 45.6 (C8), 56.2 (C10 and C12), 60.7 (C11), 71.3 (C9′), 73.6 (C7′), 101.6 (C10′), 107.0 (C2′), 108.1 (C2 and C6), 109.7 (C5′), 128.3 (C1′), 132.3 (C6′), 134.8 (C1), 137.2 (C4), 147.6 (C3′), 148.1 (C4′), 152.6 (C3 and C5), 171.4 (—O—*CO*—CH$_3$), 173.6 (C9). The mixed-melting-point test of compound (3) with authentic podophyllotoxin monoacetate proved compound (3) to be identical with podophyllotoxin monoacetate.

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